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A novel regulatory circuit in base excision repair involving AP endonuclease 1, Creb1 and DNA polymerase β

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ABSTRACT

DNA repair is required to maintain genome stability in stem cells and early embryos. At critical junctures, oxidative damage to DNA requires the base excision repair (BER) pathway. Since early zebrafish embryos lack the major polymerase in BER, DNA polymerase ß, repair proceeds via replicative polymerases, even though there is ample *polb* mRNA. Here, we report that Polb protein fails to appear at the appropriate time in development when AP endonuclease 1 (Apex), the upstream protein in BER, is knocked down. Because *polb* contains a Creb1 binding site, we examined whether knockdown of Apex affects *creb1*. Apex knockdown results in loss of Creb1 and Creb complex members but not Creb1 phosphorylation. This effect is independent of p53. Although both *apex* and *creb1* mRNA rescue Creb1 and Polb after Apex knockdown, Apex is not a co-activator of *creb1* transcription. This observation has broad significance, as similar results occur when Apex is inhibited in B cells from *apex*$^{+/-}$ mice. These results describe a novel regulatory circuit involving Apex, Creb1 and Polb and provide a mechanism for lethality of Apex loss in higher eukaryotes.

INTRODUCTION

Oxidative damage to DNA requiring the base excision repair (BER) pathway is important in normal embryological development and cell differentiation (1–4). Two critical enzymes in the pathway are AP endonuclease 1 (Apex) and DNA polymerase ß (Polb). The former makes a single nick 5′ to an abasic site in double-stranded DNA (5), while in the next step the latter inserts the replacement nucleotide(s) (5). Although the two proteins do not form a stable complex, they associate with one another during the repair process (6,7). Mouse embryos die shortly after gastrulation when Apex is knocked out (8), while zebrafish embryos develop with brain, heart and neuron abnormalities that result in termination of development later, after the primordia for all organs have formed (9). To date, no viable cultured line has been recovered from knockout mouse embryos. Furthermore, inhibition of Apex in primary cultures of Apex deficient murine B cells results in failure of class switching during lymphocyte maturation (10).

Because BER is so important in embryogenesis, we examined the major polymerase involved in the repair pathway. Although mRNA for Polb is present throughout embryogenesis, zebrafish embryos lack Polb protein before gastrulation (11). This suggests that there may be an important mechanism regulating transcription and/or translation. We asked here how this process might occur. Our results show that Apex regulates transcription of Creb1 and its binding partners, which then regulates Polb. These studies are the first to indicate a role for Apex in regulating downstream participants in BER and the first to identify a regulator for protein levels of the important transcription factor Creb1 and participants in the Creb complex.

MATERIALS AND METHODS

Mouse and fish husbandry and breeding

Wild-type zebrafish, purchased from Aquatic Tropicals (Plant City, FL), were maintained and bred as described (9,11). Homozygous p53 mutant zebrafish adults ($p53^{M214K/\ M214K}$) were the kind gift of Dr Thomas Look (12). Wild type C57BL/6 mice were housed in the

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IACUC-approved specific pathogen-free facility at the University of Massachusetts Medical School. The mice were bred and used according to the guidelines from the University of Massachusetts Animal Care and Use Committee.

Cell culture, total RNA isolation, electrophoresis, transfer and northern blotting

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). After adult fish were ground in liquid nitrogen, 50 mg homogenate was processed in 1 ml TRIzol reagent. Embryos were homogenized in TRIzol reagent by passing the embryos through a 22.5 gage needle. Total RNA was extracted as described in the manufacturer's instructions, and then subjected to phenol/chloroform purification to improve quality. For mouse B-cell cultures, spleens of WT C57BL/6 mice were dispersed and depleted of T cells, and B cells were cultured at 4×10^5/ml for 3 days with lipopolysaccharide (50 μg/ml, Sigma-Aldrich, St. Louis, MO, USA), anti-IgD-dextran (0.3 ng/ml) in the absence of BLyS with DMSO or 300 μM CRT0044876 (Maybridge Trevillet) added as previously described (10).

For northern blots, ~30 μg total RNA was resolved in a 1.2% denaturing agarose gel containing 18% deionized formaldehyde at 70 V for 3 h. After electrophoresis, RNA was transferred to nitrocellulose membranes as described in Supplementary Data for Southern blotting and stored at 4°C. To probe for *polb* mRNA, the same DNA probe used in Southern blot analysis was prepared for northern blots. The membrane was first prehybridized at 68°C by immersing the membrane in ExpressHyb™ solution for 1.5 h. Hybridization at 68°C for 3 h was followed by one wash at room temperature and a second at 55°C for 1 h each. The distribution of isotopically labeled probe was determined by phosphorImager analysis and quantitation using ImageQuant software (11).

Quantitative real-time polymerase chain reaction

Total RNA, extracted from different stage embryos and digested with RNase-free DNase, was reverse transcribed using High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (qRT–PCR) was performed as described (11) using the following thermal cycle parameters: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. The mean value of triplicate determinations was normalized to transcript levels of B-actin that served as the internal control.

Protein extraction, gel electrophoresis, transfer and western blotting

Protein extraction and western blotting were performed as described (9,11). Anti-zfApex1 antibody was prepared against zebrafish residues 140–155 by Sigma-Genosys (The Woodlands, TX, USA) (9). Unless indicated otherwise, all western blots detecting Apex were performed using this antibody. For antibody directed against human AP endonuclease 1 (hApex), we used antibody purchased from Novus Biologicals (Littleton, CO, USA). For antibody directed against Polb, we used either a mouse monoclonal anti-rat Polb antibody (Thermo scientific, Fremont, CA, USA) or a rabbit polyclonal custom antibody (21 Century Biochemicals, Marlboro, MA, USA) prepared against zebrafish Polb residues 324–339 (11). Polyclonal rabbit antibodies to detect Creb1 and Creb1 complex peptides conserved in zebrafish were obtained from Abcam Inc. (Cambridge, MA, USA) for Creb1 and p133Creb1, or from Cell Signaling (Santa Cruz Biotech Inc., Santa Cruz, CA, USA) for Crtc1, Cbp and Crem. To our knowledge there is no commercially available antiserum for Crtc3 at this time. In all cases bands of the molecular weight expected based on the sequence of the appropriate zebrafish protein were detected. Images were quantified using ImageJ software (http://rsbweb.nih.gov/ij/) and normalized to intensities of B-actin obtained with antibody purchased from GeneTex Inc. (Irvine, CA, USA).

Knockdown of selected genes by morpholino microinjection

All MOs, synthesized by GeneTools, LLC (Philomath, OR), are listed in Supplementary Table S1. Two nanoliter MO at 3 ng/nl was injected into 1–2 cell stage embryos, using phenol red as an injection indicator. It is necessary to microinject prior to the 8-cell stage so that the MO will distribute equally to all cells in the embryo. Injection volume was determined by calibration performed on a 1× 0.01 mm stage micrometer (Thermo scientific, Fremont, CA, USA). Injected embryos were raised at 28.6°C to the desired developmental stages. Phenotypes were examined daily using a Leica stereomicroscope (Bannockburn, IL, USA) and photographed or harvested for biochemistry.

Plasmid construction and capped RNA synthesis

Supplementary Table S1 lists all primers used in this study. To construct pCS2$^+$-GFP-Polb, enhanced GFP gene (eGFP) was amplified from p3E-eGFPpA vector with primer set eGFP-BamHI-For and eGFP-EcoRI-Rev and cloned into the pCS2$^+$ expression vector between the BamHI and EcoRI cloning sites. Zebrafish *polb* gene was amplified from first-strand cDNA using the primer set polb-EX-For/Rev. Zebrafish *polb* coding region was then cloned into pCS2$^+$-eGFP plasmid downstream of the eGFP gene.

To construct the pCreb1-GFP plasmid, 3040 bp of the creb1 promoter preceding the ATG start codon was amplified using *creb1* promoter primers For/Rev (CrebP-For/Rev). After digestion with XhoI and BamHI, the *creb1* promoter sequence was inserted into peGFP-N3 vector between the XhoI and BamHI sites to displace the original cytomegalovirus promoter (13). To construct pCS2$^+$-creb1, the 957 bp *creb1* open reading frame (ORF) was cloned from first-strand cDNA with the *creb1* ORF-For/Rev primer set. After digestion with EcoRI and SneBI, *creb1* ORF was cloned into pCS2+ between the EcoRI and SneBI sites.

To obtain capped RNA for eGFP tagged *polb* or for *creb1* RNA, *in vitro* transcription was performed using Ambion® mMESSAGE mMACHINE® SP6 Kit (Applied Biosystems, Austin, TX, USA), followed by purification with the MEGAclear™ Kit (Applied Biosystems). RNA (540 pg) was microinjected into 1–2 cell stage embryos. eGFP expression and embryonic development were examined daily by fluorescence microscopy. Western blot analysis was performed to confirm the overexpression of Polb protein with both zebrafish specific Polb antibody and anti-GFP antibody (Cell Signaling Technology Inc., Danvers, MA, USA).

Methyl methanesulfonate treatment

Water-injected (control), MO knockdown and *polb* mRNA rescued embryos were subjected to methyl methanesulfonate (MMS) treatment beginning at 4 hours post-fertilization (hpf). Rescue experiments were performed by co-injection of MO2 and eGFP-polb mRNA. Chronic treatment was performed by placing embryos in MMS diluted in fish water for a total of 5 days, with daily changes of medium. Embryos were examined daily for developmental progress. Death was evaluated as cessation of heartbeat and circulation on or before 5 dpf. Percent survival was recorded as surviving embryos at 5 dpf divided by total number of embryos subjected to the treatment.

RESULTS

Knocking down Polb protein results in increased sensitivity to alkylation agents

Although eggs and early zebrafish embryos lack Polb protein (11), they had ample *polb* mRNA, as shown by northern blots of RNA obtained from embryos at various stages and adult fish (Figure 1). The single band matched the 1594 bp cDNA expected from the sequence cloned from the single copy identified in zebrafish genomic DNA (Supplementary Figures S1 and S2). Expression of *polb* message peaked at the beginning of the midblastula (MBT) transition ~3 hpf, after which transcript levels decreased gradually to approximately one-eighth of the amount present in 3 hpf embryos (Figure 1B). Since Polb protein was not detected before 13 hpf (11), protein synthesis for this protein did not correlate with mRNA expression. Overexpression of Polb had no obvious morphological effect in developing embryos.

Knockdown by microinjection of morpholino oligonucleotides (MO) targeted to the translation start site (MO1) or to the junction of intron 1 and exon 2 (MO2) delayed appearance of Polb for up to 4 dpf as shown by both qRT-PCR and western blotting (Figure 2). Failure to process *polb* pre-mRNA confirmed successful knockdown (Figure 2B). After knockdown, Polb protein levels did not recover until 5 dpf. Consistent with results from cultured mammalian cells and murine embryos (11,12), there were no obvious morphological abnormalities in early knockdown embryos. Antibody staining with MF20,

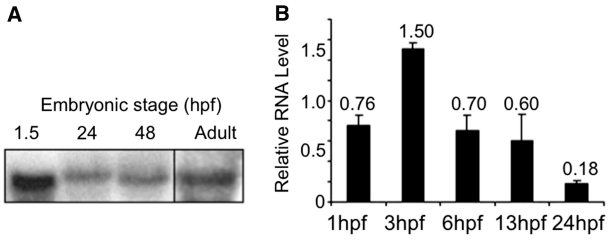

Figure 1. Messenger RNA for *polb* is plentiful in early stage zebrafish embryos. Developmental stages are indicated as hours post-fertilization (hpf). Values represent the average of 3 independent experiments ± SD of the mean. (**A**) Northern blot of total RNA extracted from different stage embryos and adults. Each lane was loaded with 30 μg total RNA and probed with α[^{32}P] dCTP labeled *polb* cDNA. (**B**) qRT–PCR quantitation of *polb* mRNA expression within the first 24 h of development. Data were generated from two independent experiments.

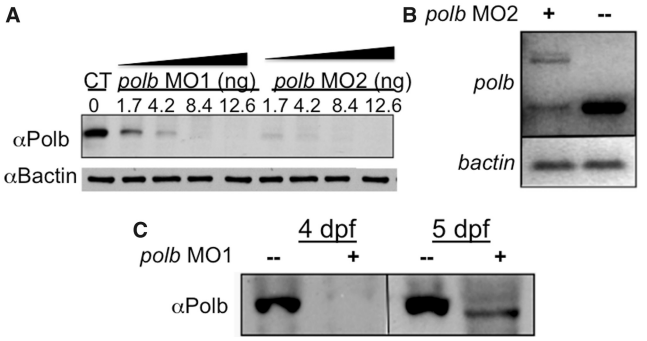

Figure 2. Both *polb* morpholinos MO1 and MO2 can effectively block Polb translation which does not resume until 5 dpf. (**A**) Western blot showing loss of Polb protein after microinjection of different concentrations of MO1 (directed against the translation start site of polb) or MO2 (directed against the intron1/exon2 splice site). Protein extracts were prepared from 1 dpf (days post fertilization) embryos. (**B**) qRT–PCR shows that MO2, a splicing blocker targeting the intron 1/exon 2 junction, produced a *polb* splice variant that retains intron1 (upper band), besides the normal transcript (lower band). B-actin, shown in the lower panel, served as control. (**C**) Western blotting confirms that after knockdown of *polb,* Polb protein does not appear until 5 dpf. Total protein from Polb knockdown embryos and controls was extracted at 4 and 5 dpf, and examined by western blot analysis for the presence of Polb protein.

which recognizes sarcomeric myosin in both skeletal and cardiac muscle, Alcian blue cartilage staining and o-dianisidine red blood cell staining all showed no difference between Polb knockdown and control embryos (data not shown). Hence, Polb did not play an indispensable role during very early morphogenesis, which is consistent with the case in early murine embryogenesis (14,15). However, 84 and 61% larvae microinjected with MO1 and MO2, respectively, failed to inflate their swim bladders. Although failure to inflate the swimbladder is a common sign of stress in zebrafish, the swimbladder is the teleost equivalent of the mammalian lung, which is defective in *polb*$^{-}$/$^{-}$ mouse knockouts, so that *polb*$^{-}$/$^{-}$ pups die shortly after birth (15). Therefore the failure to inflate may be a specific response to loss of Polb.

Although there were no major morphological alterations in development due to loss or overexpression of Polb, cell lines from Polb knockdown embryos are hypersensitive to the alkylating agent, MMS, which causes alkylation damage to DNA requiring repair by BER (16). As predicted, zebrafish embryos in which *polb* had been knocked down were more sensitive to MMS administered as a chronic exposure beginning at 4 hpf. The knockdown embryos had significantly ($P < 0.05$) lower median lethal dosage (LC_{50}) values (0.083 ± 0.003 mM) than those in untreated control embryos (0.113 ± 0.003 mM). Microinjection of ectopic eGFP tagged wild-type zebrafish *polb* mRNA along with MO2 into 1-cell stage embryos partially restored the resistance to MMS in knockdown embryos (0.096 ± 0.010 mM). MO2 did not interfere with the expression of ectopic *eGFP-zfpolb* mRNA, because MO2 blocked splicing but not transcription initiation (Figure 2B). Successful rescue was confirmed by western blot analysis using anti-GFP antibody. Therefore, loss of Polb protein in early embryogenesis resulted in blunting of DNA repair by BER.

Reduction of Apex results in loss of Polb protein via Creb1

The protein upstream of Polb in the BER pathway is Apex (5), which is likely to interact directly with Polb during BER (6,7,17). We determined the effects of reducing the levels of the BER partners on each other when we altered the level of each protein. Western blot analysis showed that knocking down Polb did not alter levels of Apex (Figure 3A). In contrast and to our surprise, knocking down Apex, the effect of which lasted for up to 4 days (Figure 3B), resulted in a substantial reduction of both Polb protein (Figure 3C, D and F) and *polb* message (Figure 3H). Recovery of Polb levels corresponded to the reappearance of Apex at ~5 dpf. These results suggest that Apex is an important regulator of its partner in the BER pathway by regulating expression of the enzyme immediately downstream from it.

Previous work has shown that Creb1 is required for *polb* transcription (18,19). Since we had just determined that Apex was important for *polb* transcription, we examined whether there might be a connection between Apex, Creb1

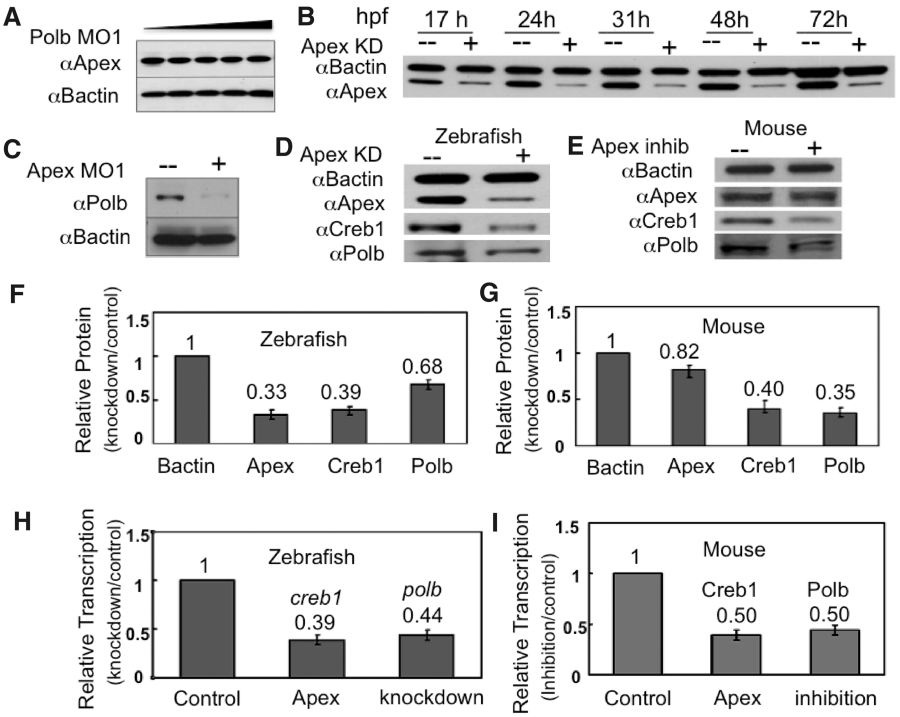

Figure 3. While knockdown of Polb does not affect Apex levels, knockdown of Apex has a profound effect on Polb and Creb1 in both zebrafish embryos and primary cultures of murine B cells. (**A**) Knockdown of Polb does not alter Apex protein levels. Extracts were prepared from 1 dpf embryos in which Polb had been knocked down by microinjection of increasing amounts of MO1 and probed by western blot analysis using rabbit polyclonal anti-Apex or anti-B actin (9). Embryos were microinjected with Polb MO1 or water; extracts were prepared at 1 dpf. (**B**) Knockdown of Apex by MO results in Apex loss for the duration of these experiments. Western blot analysis of protein extracts prepared on the indicated days was performed using anti-Apex and anti-B-actin. Loss of Apex, shown here for 3 days after knockdown, was usually detected for up to 5 dpf. (**C**) Knockdown of Apex results in failure of Polb protein to appear at the appropriate time in zebrafish embryos. Protein extracts were prepared from 3 dpf embryos. Apex hypomorphic embryos (+) were created by microinjecting 0.15 mM Apex MO at the 1–2 cell stage. (**D** and **E**) Western blotting analysis of Creb1 and Polb protein levels in Apex knockdown embryos at 24 hpf or Apex-inhibited mouse primary B lymphocytes. Note that in the latter, Apex activity is inhibited by CRT0044876 but that Apex protein is only slightly diminished. (**F** and **G**) Quantitation of western blot results in Apex knockdown embryos and Apex-inhibited mouse primary B lymphocytes. Data for (F) are the average of four independent experiments ± SD of the mean. Values for (G) are the average of three independent experiments ± SD of the mean. (**H** and **I**) Quantitative RT–PCR analysis of creb1 and polb mRNA levels in Apex knockdown zebrafish embryos and Apex-inhibited mouse lymphocytes. Loss of Apex in either mouse cells or zebrafish embryos results in diminution of both creb1 and polb mRNA. The values for H are the average of five independent experiments ± SD of the mean, while the values for I are the average of three independent experiments ± SD of the mean.

and Polb. Indeed, microinjection of MO directed against the Apex translation start site resulted in loss not only of Apex protein and failure of Polb to appear in a timely fashion, but also of ~50% Creb1 protein and message at 24 hpf (Figure 3D, F and H).

To confirm the relationship between Creb1, Apex and Polb, we first rescued Apex knockdown by co-injection of MO directed against the Apex translation start site with or without capped mRNA encoding WT human AP endonuclease 1 or the catalytically inactive site-directed mutant containing a triple mutation in the active site (Y171F-P173L-N174K) (Figure 4A). Although this mutant is enzymatically inactive, it retains conformation similar to the native enzyme (20). The gene for the human protein was chosen for rescue experiments, because the knockdown MO designed to target the ATG start site of the zebrafish message does not interfere with expression of the human message (9). Extracts prepared at 24 hpf were examined for the presence of *apex*, *creb1* and *polb* message and protein (Figure 4). Results were entirely consistent with earlier findings (9), that rescue required the endonuclease function of the protein, since mRNA for WT human Apex but not the active site mutant lacking endonuclease activity resulted in rescue. We then overexpressed *creb1* mRNA with or without knockdown of Apex. We observed increased *creb1* mRNA in control embryos that had not been microinjected with MO and entirely restored loss of *creb1* mRNA in embryos in which Apex had been knocked down (Figure 4C). Furthermore, overexpression of *creb1* message resulted in enhanced levels of *polb* message when Apex had not been knocked down and restored *polb* message in Apex knockdowns.

In some systems, PKA-mediated phosphorylation of Creb1 promotes Creb1 nuclear localization and complex binding (21–23). To determine whether loss of Polb was mediated through PKA, we used western blot analysis to examine whether Creb1 phosphorylation was altered at Ser^{111} when Apex was knocked down. (Note that Ser^{133}

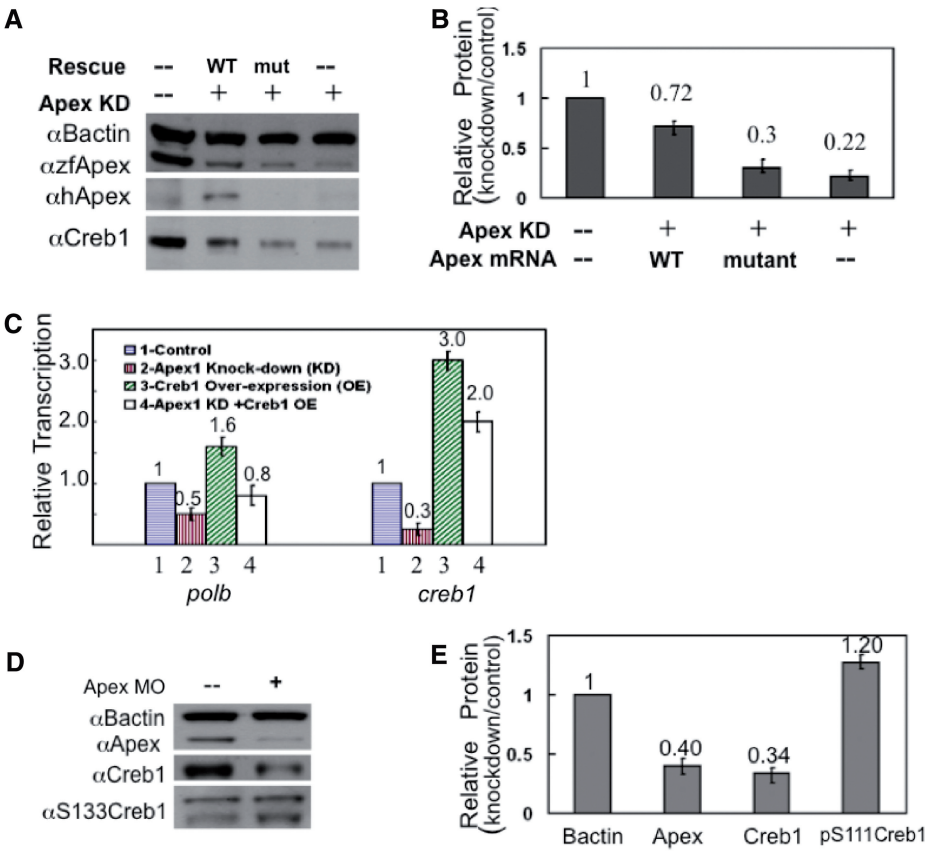

Figure 4. Rescue experiments confirm the role of Apex and Creb in maintaining Polb; Apex knockdown does not alter Creb1 phosphorylation at 24 hpf. (**A**) Microinjection of capped mRNA encoding WT human AP endonuclease 1 (hApex) rescued both Apex and Creb1 levels in Apex knockdown embryos. Note that antiserum directed against peptide 140–155 of the zebrafish protein (αzfApex) was able to recognize both WT and mutant hApex proteins, while antibody against hApex (αhApex) recognized only WT hApex protein. (**B**) Quantitation of relative Creb protein levels from (A). These data represent the mean ± SD of three independent experiments. (**C**) Microinjection of capped *creb1* mRNA rescued *creb1* and *polb* transcription in early zebrafish embryos. Apex was knocked down by microinjection of TS-MO in the presence or absence of *creb1* capped RNA at the 1–2 cell stage. Total RNA was prepared from 24 hpf extracts. Messenger RNA levels for the indicated genes under the different conditions relative to *b-actin* transcription were determined by qRT–PCR. Data were then normalized to the mRNA level found in control extracts from embryos microinjected with water. This panel shows that co-injection of *creb1* mRNA restores *polb* mRNA levels. Values represent the average of three independent experiments ± SD of the mean. (**D**) Knockdown of Apex did not alter Creb phosphorylation at 24 hpf. Western blot analysis of different forms of Creb1 protein in 24 hpf embryos. Note that despite the fact that Creb1 itself was diminished, p133Creb remained unchanged at 24 hpf. (**E**) Quantitation of western blot results shown in Figure 4D. The values are the average of three independent experiments ± SD of the mean.

is the corresponding residue in the human ortholog.) Phosphorylation at that residue was not altered at 24 hpf (Figure 4D and E). Consequently, the ratio of $pSer^{111}$ relative to remaining Creb was increased an average of 2-fold at 24 hpf. The elevated level of phosphorylated Creb1 was not maintained, however, since by 48 hpf phosphorylation at Ser^{111} had decreased to 60% of control levels. Creb1 can also be phosphorylated at two additional serine residues, Ser^{107} and Ser^{120}, which are equivalent to Ser^{129} and Ser^{142} in the human protein. Phosphorylation at these two residues decreased commensurately with the loss in Creb1 (data not shown). Thus, any change in PKA activity was secondary to the initial loss of Creb1 protein.

Apex regulates other members of the Creb complex

To examine whether participants in the Creb1 complex (24,25), such as Creb response element modulator (Crem), Creb binding protein (Cbp), and Creb regulated transcription coactivator (Crtc) 1 and 3, were affected by loss of Apex, we performed qRT–PCR and western blot analysis on extracts from 24 hpf control and Apex knockdown embryos. Loss of Apex resulted in substantial (40–85%) loss of *crem*, *cbp* and *crtc 1* and *3* mRNA (Figure 5A). Corresponding protein levels were reduced up to 65% (Figure 5B and C). Therefore, Apex regulates not only Creb1 levels but also levels of Creb binding partners.

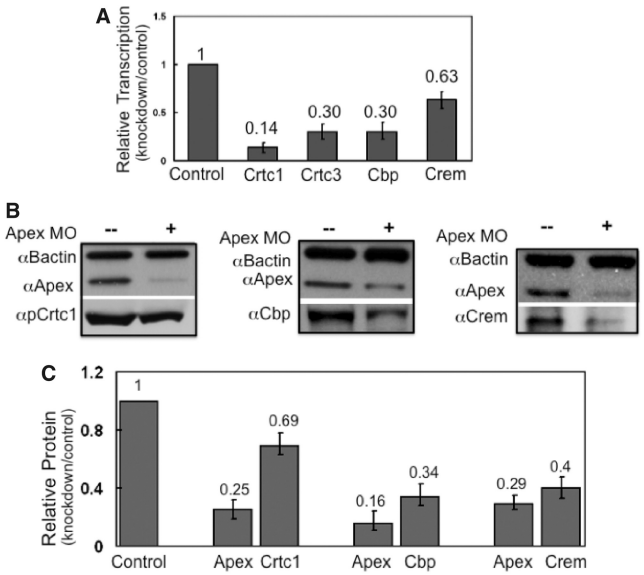

Figure 5. Apex regulates proteins in the Creb-dependent transcription complex. (**A**) Transcripts of proteins that participate in the Creb complex were measured by qRT–PCR in control and Apex knockdown 24 hpf embryos. Messenger RNA for Crtc1, Crtc3, Cbp and Crem were examined using primers described in Supplementary Table S1. Data are presented as ratio of control normalized for *b-actin* message. Values are the average of three independent experiments ± SD of the mean. (**B**) Western blot analysis showed that proteins in the Creb complex (Creb1 itself, Crtc1, Cbp and Crem) are decreased when Apex is knocked down. We did not quantify Crtc3, as there is no commercially available antiserum. (**C**) Quantitation of western blot results of Creb complex proteins in 24 hpf control and Apex knockdown embryos. Values are the average of three independent experiments ± SD of the mean.

Apex regulation of Creb1 is independent of p53

Because apoptosis is the most pronounced result of knocking down Apex in most cultured cells, we asked whether p53 might be involved in loss of Creb. We repeated the knockdown experiments in homozygous p53 ($p53^{M214K/\ M214K}$) mutant zebrafish. These mutant fish fail to upregulate p21, do not arrest at the G1/S checkpoint and fail to undergo radiation-induced apoptosis (12). Since knockdown of Apex resulted in diminution of Creb in the absence of p53 (Figure 6A and B), we conclude that Creb regulation by Apex is p53 independent. Our results may explain some of the p53 independent effects in mammalian cells exposed to MMS (26).

Apex is not a coactivator of Creb1

Since the mRNA for many, if not all, members of the Creb1 complex and for Polb contains a Creb1 binding site, we asked whether Apex was a co-activator of Creb1. To that end, we inserted the 3040 bp sequence upstream of the *creb1* gene in front of the coding sequence for green fluorescent protein (GFP) to obtain pCreb1P3040-eGFP (13). After microinjection of 50 pg pCreb1P3040-eGFP per embryo with or without the MO to knockdown Apex, we followed the appearance and localization of GFP (Figure 6C and D). GFP in control embryos appeared in selected regions of the head and along the trunk. After knockdown of Apex, the fluorescence was intensified, while the location remained unaltered. Western blot results confirmed knockdown of Apex and increased GFP. Therefore, we conclude that Apex is not a co-activator of the *creb1* promoter, or at least not one acting within 3000 bp of the Creb binding site.

Loss of *creb1* and *polb* message and protein due to reduction of Apex is not limited to teleosts

Murine primary B lymphocytes divide more slowly and fail to undergo class switching when Apex is inhibited with 7-nitro-1H-indole-2-carboxylic acid (CRT0044876) (10). To determine whether the results with zebrafish embryos were unique to embryos or to fish, we examined changes in *creb1* and *polb* message and protein in primary cultures of murine B lymphocytes cultured in the presence or absence of 300 μM CRT0044876. We observed the loss of *polb* and *creb1* message and protein after inhibition of Apex activity (Figure 3E, G and I), although the amount of Apex protein in B lymphocytes changed little and viability was not substantially affected. Therefore, Apex regulates Creb1 function in higher vertebrates including mouse B cells and zebrafish embryos. Creb1 is important at many stages of B cell development and for B cell survival (27). Furthermore, these data are consistent with the observation that $Apex^{+/-}$ mice have reduced levels of PolB (28).

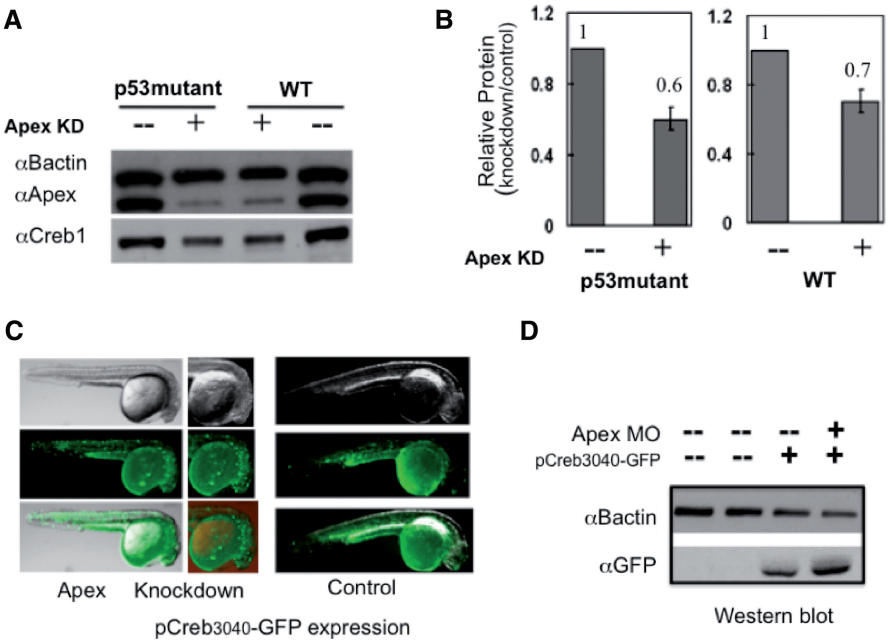

Figure 6. Effects of Apex knockdown are independent of p53; Apex is not a co-activator of Creb1. (**A**) To examine whether loss of Creb was p53-dependent, we performed Apex knockdown in WT and $p53^{M214K/\ M214K}$ fish. Cell extracts were prepared at 24 hpf and analyzed for Apex and Creb by western blotting. Apex knockdown resulted in similar loss of Creb in both WT and $p53^{M214K/\ M214K}$ fish. (**B**) Quantitation of relative Creb protein levels from (A). The values are the average of 3 independent experiments ± SD of the mean. (**C**) To examine whether Apex is a co-activator of *creb* transcription we microinjected pCreb1P3040-eGFP with or without Apex knockdown and examined the embryos by fluorescence microscopy or western blot analysis. Distribution and intensity of GFP increased somewhat after Apex knockdown. Note the regions of greatest intensity in 24 hpf embryos in the head region, which remained unaltered by Apex knockdown. (**D**) Western blot, using rabbit antiGFP antibody, confirmed increased levels of GFP protein after knockdown of Apex.

DISCUSSION

We have provided conclusive evidence for a novel regulatory circuit in BER, one in which AP endonuclease 1 regulates the level of *creb1* transcript and Creb1 protein in zebrafish early embryos and primary cultures of murine B cells. It also regulates levels of mRNA and protein levels of other proteins in the Creb1 complex, including Cbp, Crem and Crtc1 and 3 and thereby regulates the level of Polb, its partner in the BER pathway. Figure 7 is a cartoon summarizing our results. Since each of the proteins in the Creb complex is reduced by 60–80% when Apex function is reduced, the opportunity to form the Creb complex could be reduced by as much as 95%. Therefore, transcription of other genes that are positively regulated by Creb1 could be reduced to <5% control levels. Since reduction in the individual proteins is commensurate with reduction in mRNA levels, the primary effect is at the transcriptional or message stability level, rather than at the level of protein stability. These data provide the first evidence for a protein that regulates *creb1* transcription and Creb1 protein levels. Since Creb1 regulates over 100 pathways (29–33), AP endonuclease 1, first known for its participation as an endonuclease in the base excision DNA repair pathway, serves as a driver gene for a master regulator of multiple major pathways in higher eukaryotic cells.

Loss of Apex also results in loss of *polb* mRNA, which corresponds to delayed appearance of Polb in

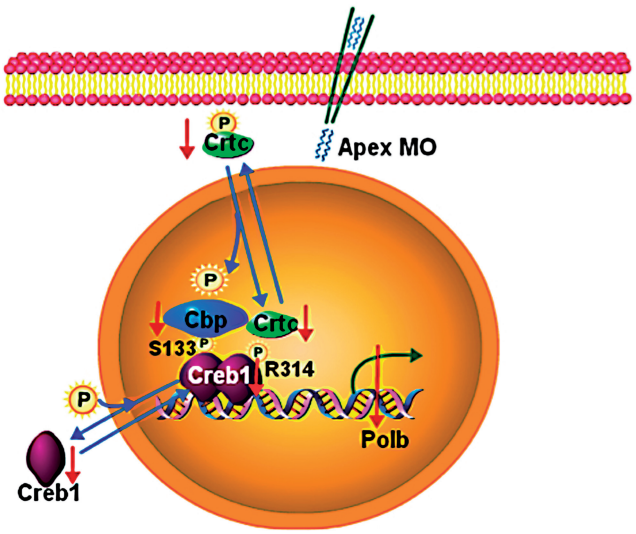

Figure 7. Cartoon showing how Apex might regulate DNA polymerase β via the Creb complex. Note that the numbering of residues on Creb1 is that of the human homologue in order to avoid confusion in the literature. However, Ser^{133} in the human protein is Ser^{111} in the zebrafish protein. Similarly, Arg^{314} in the human protein, which binds Crtc1 when it is phosphorylated, is Arg^{291} in the zebrafish protein. These data indicate that Apex maintains levels of Creb1 and its partners without affecting PKA-mediated phosphorylation of Creb1. Consequently, in the absence of Apex levels of Creb1 complex diminish and Creb-mediated transcription is altered.

development. Since Creb is required not only for transcription of *polb* (18,19) but also for transcription of *sp1* and *NFκB*, two additional transcription factors required for *polb* transcription, failure of Polb to appear in a timely fashion is likely to be the result of diminution of Creb and the members of its complex. Because Apex is required for the synthesis of Polb, the next protein in the BER pathway, Apex can be designated a 'feed forward activator' (34–36). However, the presence of Apex is not sufficient to ensure the synthesis of Polb, since zebrafish eggs and early-stage embryos contain a surfeit of Apex protein and *polb* mRNA but lack detectable Polb protein. Other regulators must also be involved in preventing synthesis of Polb protein in early embryos.

Indeed, other regulators are likely to be involved in selecting which transcripts are up- or down-regulated after Apex reduction, since some Creb1-dependent transcripts are upregulated under knockdown conditions. These results are in keeping with studies that show that which Creb1-dependent genes are altered at any time depends on other members of the complex, such as Cbp, Torc and/or p300 (37–39).

Although loss of Apex is ultimately lethal for both mouse and zebrafish embryonic development, our findings reinforce the requirement for endonuclease activity rather than any redox activity for successful embryogenesis and cell survival. Not only does the WT zebrafish protein lack the requisite cysteine residue which renders it redox inactive in standard electrophoretic mobility shift assays (9,40) but also rescue in knockdown zebrafish embryos requires the endonuclease function. Furthermore, development of mouse embryos is not affected when the corresponding redox-relevant cysteine (Cys^{64}) is converted to alanine (41). How might the endonuclease activity regulate *creb* transcription/stability? Since Apex does not make a Schiff base when it cleaves an AP site, the mechanism by which it regulates *creb1* levels is different from that of Ku's cleavage of AP sites in proximity to double strand breaks in DNA (42). Apex does have the ability to cleave RNA (43,44), which requires the same active site as cleavage of an AP site in DNA (43). Although the requirement for an enzymatically active site is consistent with our knockdown/rescue results, how *loss* of an RNase activity might account for *reduction* of *creb1* mRNA is not clear. Regardless of the mechanism, that loss of Apex results in the loss of Creb1 and its complex partners, which regulate multiple interlocking pathways (45–47), can account for lethality in higher eukaryotic cells and embryos.

Because zebrafish embryos continue to develop through hatching without a functional heart, we were able to follow important pathways and cell types that depend on Apex. These studies could not have been done in mammalian embryos, since abnormal cardiac development in mouse embryos leads to resorption shortly after gastrulation. Nor could they could have been done in cultured cells, since most cultured cells undergo apoptosis when Apex levels are reduced to the levels obtained here. The finding that Apex regulates Creb1 levels in the absence of *p53* implies that the effects of Apex reduction may be independent of p53-mediated apoptosis in cultured cells. Furthermore, since Creb1 regulates expression of the antiapoptotic genes *mcl-1* and *bcl-2 in vitro* in B cells (48), these data suggest that Apex regulation of Creb may be a mechanism to protect against cell death, perhaps by preventing apoptosis to allow time for DNA to be repaired. This mechanism could be particularly critical for cephalic, neural and cardiac tissues that comprise the Apex knockdown phenotype and are critically sensitive to oxidative damage.

Besides serving as a driver for Creb, Apex has been proposed to participate in regulation of transcription as a trans-acting transcription factor (49–52). Apex also associates with HIF-1α, p300 and STAT3 in a complex to regulate VEGF transcription (38,53) and enhances binding of Egr-1 to its cognate sequence, which in turn activates PTEN expression (54,55). Although our data indicate that Apex is unlikely to serve as a co-activator of Creb1, both Egr1 and HIF1α contain Creb binding sites and the effect could be mediated through the Creb complex.

Creb1 anchors a transcription complex that regulates multiple pathways in cell physiology. Specificity is likely to be conferred by individual factor(s) completing the complex. Despite the intense focus on Creb1 over the years, how its protein level is regulated was not evident until our study. We have clearly demonstrated for the first time that AP endonuclease 1 regulates not only Creb1 protein levels but also the protein levels of other Creb complex members. In so doing, it controls the protein levels of DNA polymerase ß, its downstream partner in the BER pathway. This finding opens new horizons to further understand the importance of the multifunctional protein, Apex, in regulating cell physiology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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